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Expression profiles of 10,422 genes at early stage of low nitrogen stress in rice assayed using a cDNA microarray

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Abstract

Development of crop varieties with high nitrogen use efficiency (NUE) is imperative for sustainable agriculture. Understanding how plant genes respond to low N stress is essential for formulating approaches to manipulating genes for improving NUE. In this study we analyzed the expression profiles of an indica rice cultivar Minghui 63 at seedling stage at 20 min, 1 and 2 h after low N stress with the normal N as the control, using a microarray of 11,494 rice ESTs representing 10,422 unique genes. While no significant difference was detected in the leaf tissue, a total of 471 ESTs were detected as responsive to low N stress in the root tissue with 115 ESTs showing up-regulation and 358 ESTs showing down-regulation. The analysis of expression profiles after low N stress identified following patterns: (1) the genes involved in photosynthesis and energy metabolism were down-regulated rapidly; (2) many of the genes involved in early responses to biotic and abiotic stresses were up-regulated while many other stress responsive genes were down-regulated; (3) regulatory genes including transcription factors and ones involved in signal transduction were both up- and down-regulated; and (4) the genes known to be involved in N uptake and assimilation showed little response to the low N stress. The challenges for future studies are to characterize the functional roles of the low N stress responsive genes in N metabolisms, including the large number of genes presently with unknown functions.

Introduction

Nitrogen is a crucial plant macronutrient and is needed in the greatest amount of all mineral elements required by plants. It is a constituent of numerous important compounds, including amino acids, proteins (enzymes), nucleic acids, chlorophyll and several plant hormones. It comprises 1.5–2% of plant dry matter and approximately 16% of total plant protein (Frink *et al*., 1999).

In the last half a century, the global use of N fertilizer increased 10-folds in order to increase crop productivity (UNEP, 1999), as a consequence of the fact that most of the high yielding varieties of the major crops developed in the last several decades have high demands of N and other nutrients, as well as optimal cultivation conditions. Plants consume much less than half of the fertilizers applied (Frink *et al*., 1999; Socolow, 1999), while majority of N fertilizers was lost to the atmosphere or leached into groundwater, lakes and rivers, which causes increasingly severe pollutions to the environments. United Nations Environment Programme recently reported that,

worldwide, N pollution along with water shortage and global warming pose the main threats to human survival and the environment (UNEP, 1999). Moreover, fertilizer application has now become the major cost in crop production, which greatly affects the income of the farmers. Thus, developing crops that are less dependent on the heavy application of N fertilizers is essential for the sustainability of agriculture. Technically, this means the development of crop varieties that can manage sufficient uptake of N nutrient in soils with low N concentration (high uptake efficiency), and can make the best use of the N nutrient that the plant has absorbed from the soil for producing the products (high utilization efficiency).

The main structural elements of N uptake and assimilation pathways in higher plants have been well documented. First, nitrate and ammonium are absorbed by the plant via a variety of transporters that are divided into high-affinity transport systems (HATS) and low-affinity transport systems (LATS). The HATS mediate most of the uptake activity when the N concentration is lower than 1 mM, and the LATS are responsible for the main uptake when the N concentration is increased above 1 mM (Forde and Clarkson, 1999; Glass *et al*., 2001; Williams and Miller, 2001). Two families of nitrate transporter genes, *Nrt1* and *Nrt2*, have been identified that encode the LATS and HATS for nitrate respectively (Crawford and Glass, 1998; Forde, 2000; Galvan and Fernandez, 2001). While the molecular basis of LATS for ammonium is not very clear, it is known that members of *Amt1* gene family encode the HATS for ammonium (Gazzarini *et al*., 1999; Howitt and Udvardi, 2000). After uptake into the plant, the nitrate is reduced to nitrite catalyzed by nitrate reductase (NR) and then to ammonium by nitrite reductase (NiR) (Campbell, 1988). The primary assimilation takes place both in shoots and in roots, and ammonium is incorporated into organic molecules by the Gln synthetase (GS) and Glu synthase (GOGAT) pathway (Lam *et al*., 1996; Campbell, 1999; Hirel *et al*., 2001).

Knowledge has also been accumulating regarding the response of plants to low N conditions. In the unicellular green alga *Chlamydomonas reinhardtii*, deprivation of N source initiates the program of sexual differentiation (Treier *et al*., 1989). In higher plants N deficiency results in reduced growth, gradual chlorosis of older leaves, followed by abscission. Anthocyanins are often synthesized in the stems, petioles, and leaf veins in N deficiency plant caused by excess carbohydrates accumulation (Martin *et al*., 2002). Also observed adaptations to N deficiency are altered root architecture, an increased root to shoot ratio and an increased root surface (Marschner *et al*., 1986). Application of nitrate to restricted part of roots can stimulate the lateral roots proliferation and growth (Granato and Raper, 1989; Zhang and Forde, 2000), but a general application of high nitrate concentration stimulates the shoot growth accompanied by inhibiting the root growth and delaying flowering and senescence (Bernier *et al*., 1993; Stitt, 1999).

Microarray technology is a useful tool for analyzing genome-scale gene expression (Schena *et al*., 1995). Recently, this technology has been applied in identifying pathways involved in light control (Tepperman *et al*., 2001; Ma *et al*., 2002) and the circadian clock (Harmer *et al*., 2000), and in expression profiles of plant genes under environmental stresses, such as low phosphate nutrition (Hammond *et al*., 2003; Wu *et al*., 2003), pathogen infections (Maleck *et al*., 2000), as well as cold, salt and drought treatments (Kawasaki *et al*., 2001; Seki *et al*., 2001, 2002). There have also been reported studies of nitrate response reactions induced by re-supply of nitrate, after using ammonium as the N source or with deprived of N, which identified large numbers of genes in *Arabidopsis*, including genes that are directly involved in nitrate transport, nitrate reduction and nitrite reduction, ammonium assimilation, and generation of NADPH through the oxidative pentose phosphate pathway (Wang *et al*., 2000, 2003, 2004; Price *et al*., 2004; Palenchar *et al*., 2004; Scheible *et al*., 2004).

However, knowledge is still lacking concerning gene expression and regulation of the plants in response to low N stress as frequently occurring in agricultural field conditions, while such knowledge is essential for formulating strategies for manipulating the genetic architecture of the plants to improve the N use efficiency (NUE). It is also essential to identify the signal transduction pathways and the regulatory elements that function to regulate the genes involved in the N uptake and assimilation pathways.

Rice has now become a model for genomic research of monocot species, because of its small

genome size, and the near completion of genome sequencing project (Feng *et al*., 2002; Sasaki *et al*., 2002; The Rice Chromosome 10 Sequencing Consortium, 2003). Rice is also the staple food for approximately a half of the world population. The amount of N fertilizer loss in rice fields is greater than in other crops (Zhu, 2000), loss of as much as 70% of the applied N fertilizers was reported in high yielding rice fields in China. Thus characterization of responses of rice genes to low N stress may make a good starting point for improving the NUE of the crop.

In this study we analyzed expression profile of rice seedlings at early stage of low N stress using a cDNA array containing 11,494 ESTs likely representing 10,422 unique genes. The objective was to characterize the expression patterns of the genes representing a large segment of the rice genome in response to low N stress, with the hope to advance understanding of the physiological and biochemical processes underlying the response to low N stress. It is expected that information obtained in such a study will help the development of approaches to manipulating the genes for NUE improvement of the rice crop.

Materials and methods

Plant materials and stress treatments

Seeds of the rice cultivar Minghui 63 (*Oryza sativa* ssp. *indica*) were germinated and grown hydroponically in nutrient solution containing 1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.17 mM $NaSiO_3$, 50 μm Fe-EDTA, 0.06 μM $(NH_4)_6Mo_7O_{24}$, 15 μM H_3BO_3, 8 μM $MnCl_2$, 0.12 μM $CuSO_4$, 0.12 μM $ZnSO_4$, 29 μM $FeCl_3$, 40.5 μM Citric acid, pH 5.5 (Yoshida *et al*., 1976). The culture solution was refreshed every 3 days. At the emergence of the fourth leaf, the seedlings were transferred into either a nutrient solution with the N concentration reduced to 0.24 mM NH_4NO_3 for stress treatment or a nutrient solution with the normal N concentration as the control. The concentration of the low N solution was determined based on our preliminary experiment, under which condition stress symptom (yellow leaves and fewer tillers) became visible within 9 days of low N treatment. Roots and shoots were harvested separately after 20 min, 1, and 2 h after the treatment, frozen in liquid nitrogen and keep at −70 °C until RNA isolation. The planting and harvesting were conducted twice with an interval of approximately 1 month for two biological repeats.

RNA isolation and blot analysis

Tissue samples removed from freezer were ground in liquid nitrogen. Total RNA was isolated using Trizol reagent (GIBCOL/BRL) according to the manufactures' instructions. For Northern blotting, total RNA (15 μg) was separated by electrophoresis on MOPS-formaldehyde agarose gel and blotted onto Hybond N^+ nylon membrane with 10×SSC as the transfer buffer. Probes were amplified by PCR using cDNA as templates with M13 forward and reverse primers, and labeled using the hexamer labeling method (Feinberg and Vogelstein, 1983). The filter was hybridized at 65 °C overnight in hybridization buffer containing 0.14 M NaH_2 PO_4, 0.36 M Na_2 HPO_4, 7% SDS and 1 mM EDTA. The filter was washed once in 2×SSC and 0.1% SDS for 10 min at 65 °C, and once in 0.5×SSC and 0.1% SDS for 15 min at 65 °C.

cDNA microarray preparation

A total of 11,494 unique sequences (Zhang *et al*., 2005) were selected from a normalized cDNA library of approximately 62,000 clones that were prepared by mixing approximately equal numbers of clones from cDNA libraries individually constructed for 15 different tissues of Minghui 63, and normalized by hybrid arrest with the genomic DNA (Chu *et al*., 2003). Inserts of the cDNA clones were amplified by PCR with the M13 forward and reverse primers. PCR products were precipitated with isopropanol, resuspended in 40 μl 50% DMSO, and visualized on 1% agarose gel to ensure the quality and quantity. Approximately 92.8% of the PCR products had a single band, 6.5% had two bands, and 0.7% had three or more bands. The average sizes of cDNA inserts were 1.4 ± 0.36 kb (Chu *et al*., 2003). PCR products with concentrations of 200 ± 24.5 ng/μl were arrayed onto poly-L-lysine-coated glass slides (Corning) using a GMS417 arrayer from Genetic MicroSystems (Woburn, MA) with the spot

volume of 0.5 to 1.0 nl per pin, which amounts to 0.1–0.2 ng DNA per spot in the slide. The 11,494 clones, together with a clone of porcine glyceraldehyde 3-phosphate dehydrogenase gene repeated 12 times as the negative control and a clone of a rice actin gene repeated 14 times as the positive control, were arrayed on two slides (Corning GAPS II Coated Slides) with 5760 spots on each slide (http://redb.ricefgchina.org/mged/lxm/). The slides were kept in room temperature for 48 h, and then in 80 °C for 4 h.

It should be noted that the classification of unique sequences was based on the available information at the time when the experiment was performed, and a fraction of them may not be real unique sequences.

Fluorescent probe preparation

For probe preparation, mRNA was purified from each tissue by using Dynabeads oligo$(dT)_{25}$ from Dynal A.S. (Oslo, Norway). About 1.0 μg mRNA was reverse transcribed in the presence of aa-dUTP (Amersham Pharmacia). The reverse transcription reaction was performed in 40 μl solution containing 1×Superscript II reverse transcriptase buffer (Life Technologies, Grand Island, NY), 10 mM DTT, 4 μg of anchored oligo (dT) 20mer, 400 units of Superscript II reverse transcriptase, 40 units of RNAsin (Promega, Madison, WI), 200 μM aa-dUTP, 500 μM each dATP, dCTP and dGTP, and 200 μM dTTP. After incubation at 42 °C for 1 h, another 400 units of Superscript II reverse transcriptase were added and incubated for another 1 h at 42 °C. The reaction was stopped by incubating at 94 °C for 3 min. The reaction mixture was treated with 2 units of RNase H for 10 min at 37 °C to degrade RNA, and with 1.0 μg RNase A for 10 min at 37 °C and then purified with a microcon YM-30 (Millipore). The revere transcription products for the stress and control treatments were labeled with Cy3 or Cy5 fluorescent dyes respectively by adding the dyes into the reaction products and incubating at room temperature in the dark for 1.5 h. The Cy3- and Cy5-labeled samples were combined and purified with a microcon YM-30, with the volume adjusted to 4.2 μl, to which 0.6 μl 20×SSPE, 0.6 μl blocking solution, and 9.6 μl hybridization solution were added to a total volume of 15 μl. The probe was denatured at 95 °C for 2 min, cool on ice, centrifuged in a microfuge at 14,000 rpm for 4 min to pellet any particulate matter, and then about 15 μl of the probe solution were used for hybridization.

Hybridization, washing and scanning

About 15 μl of prehybridization buffer was placed onto the center of a cover slip, and then the center of the arrayed slide was placed over the cover slip to avoid forming bubbles. The slide was inverted and placed in a chamber, which was incubated in an air bath set at 76 °C for 5 min then at 50 °C for 30 min. After prehybridization, slides were washed in double de-ionised water for 2 min, 70% ethanol for 2 min, ethanol for 2 min and spun at no more than 1000 rpm for 2 min for drying. Hybridization was carried out at 42 °C for 16 h. After hybridization, slides were washed twice with 2×SSC with 0.1% SDS for 10 min, 0.2×SSC with 0.1% SDS for 10 min twice, 0.2×SSC for 10 min twice, 0.02×SSC for 10 min and then spun at no more than 1000 rpm for 2 min for drying. Slides were scanned with a GMS418 Array Scanner (Genetic MicroSystems, Worburn, MA) by two separate laser channels for Cy3 and Cy5 emissions.

We performed forward and reverse labeling for each time point. In the forward hybridization, the mRNA from low N stressed tissue was labeled with Cy3 and the control tissue with Cy5, these labeled probes were mixed in equal amounts and hybridized with the cDNA chip. In the reverse hybridization, the mRNA from stressed tissue was labeled with Cy5 and the control tissue with Cy3. Each of the hybridizations was performed with two repeats using independent samples from two different plantings with a time interval of approximately a month, resulting in four replications for each time point, except that 1 of the 2 slides for 1 h was replicated three times.

Data analysis

Spot intensities from scanned slides were quantified with Imagene 4.2 software (Biodiscovery), in which grids were automatically placed and manually adjusted to ensure optimal spot recognition. The signal of the spot was measured as the mean of pixels within a circle surrounding the spot and the local background in a three-pixel-wide torus that is one pixel away from the fixed circle. After the poor spots were eliminated according to the

standard as described (http://redb.ricefgchina.org/mged/lxm/), the signal was normalized by setting the total signals of Cy3 and Cy5 equal. The signal mean ratio of stressed/control was generated on the basis of normalized signals and used as a relative measurement to determine the relative level of gene expression.

Two criteria were adopted for claiming "significant" differences in the level of gene expression between low N treatment and control. For establishing the first criterion, a "yellow" experiment was conducted in which the same mRNA sample was labeled with Cy3 and Cy5 respectively, and hybridized with a glass slide containing 5760 cDNA clones. In this hybridization, the expected signal ratios of Cy5/Cy3 for all the spots should be unity; signal ratios larger or smaller than unity should be regarded as experimental error. For an experiment with four independent replications, the error rate for the signal ratio of a clone to be simultaneously above the upper 2.5 percentile or below the lower 2.5 percentile would be $(0.05)^4$. In this experiment the upper- and lower-2.5 percentiles of the spots in the distribution curve were −1.36 and 1.41 respectively (Figure 1). In the analysis, we used 1.5 and −1.5 as the critical points for declaring up- or down-regulations and a sequence was regarded as showing altered expression only when the ratios of all the four replicates were simultaneously larger than 1.5 or smaller than −1.5, which further reduced the probability of false positive. As the second criterion, the log-transformed normalized signals of low N stress and control were compared using *t*-test at the 0.005 probability level (Dudoit *et al*., 2002; Tsai *et al*., 2003). Differential expression was claimed for a sequence only when the difference of the expression level under low N stress and control conditions simultaneously met both criteria. Jointly, application of these two criteria would reduce the false positive rate to essentially zero.

Functional categories of the differentially expressed genes were assigned based on homology with genes of known functions using the GO program (Gene Ontology Consortium, 2001).

Results

To assess the reproducibility of the hybridization data, correlations based on the signal intensity of low N stress and control were calculated for data resulting from the dye swap within each biological repeat (Table 1). Correlation coefficients between the data of dye swap varied from 0.913 to 0.990 with an average of 0.957.

To confirm the results of microarray analysis, 9 clones representing different expression patterns

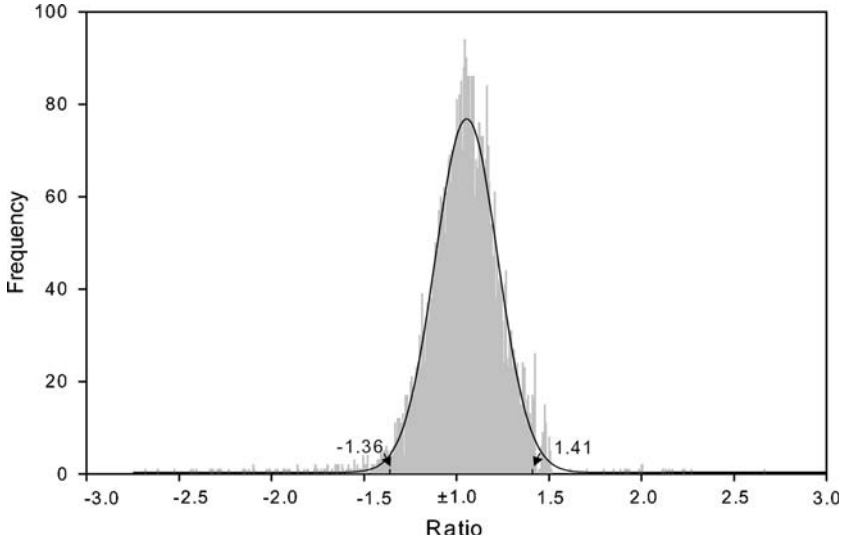

Figure 1. Distribution of the ratios of the Cy5/Cy3 in the "yellow experiment". When the ratio is smaller than 1.0, the inverse of the ratio is taken and multiplied by −1 to make the curve an approximate normal distribution. The arrows indicate the lower and upper 2.5 percentiles of the curve (−1.36 and 1.41 respectively).

Table 1. Correlations of hybridization signal intensities between dye-swap (technical replication) in each of the two biological repeats at the three time points after low N stress.

	20 min		1 h		2 h	
	Repeat 1	Repeat 2	Repeat 1	Repeat 2	Repeat 1	Repeat 2
Control	0.934	0.913	0.962	0.990	0.971	0.961
Low N	0.942	0.917	0.971	0.984	0.967	0.977

were selected for RNA gel-blot analysis. The RNA gel-blot results are generally consistent with the results from microarray data (Figure 2).

Overall features of the low N stress responsive expression profile

No significant gene expression changes were detected in shoots at any of the three time points after stress (date not shown). We also took samples from seedlings stressed with low N for 24 and 48 h, again no obvious differences were observed in the hybridization signals of the slides. This seems reasonable, since plant vacuoles in the leaves have large storage capacity for nitrogen (Van der Leij *et al.*, 1998).

In contrast, significant inductions in gene expressions were observed in root as early as 20 min after stress treatment. Since our primary interest in this study is in the early stage response of the rice seedlings to low N stress, we thus focused our analysis of gene expression profiles in root.

A total of 10,595 clones produced hybridization signals according to the criteria used in this study, and 471 genes exhibited alteration in expression in response to low N stress in at least one of the three time points. The total number of down-regulated genes 358 is much larger than that of up-regulated genes 115 (Figure 3). Among the up-regulated genes, 19, 40 and 75 genes showed elevated level of expression at 20 min, 1 and 2 h, respectively. And among the down-regulated

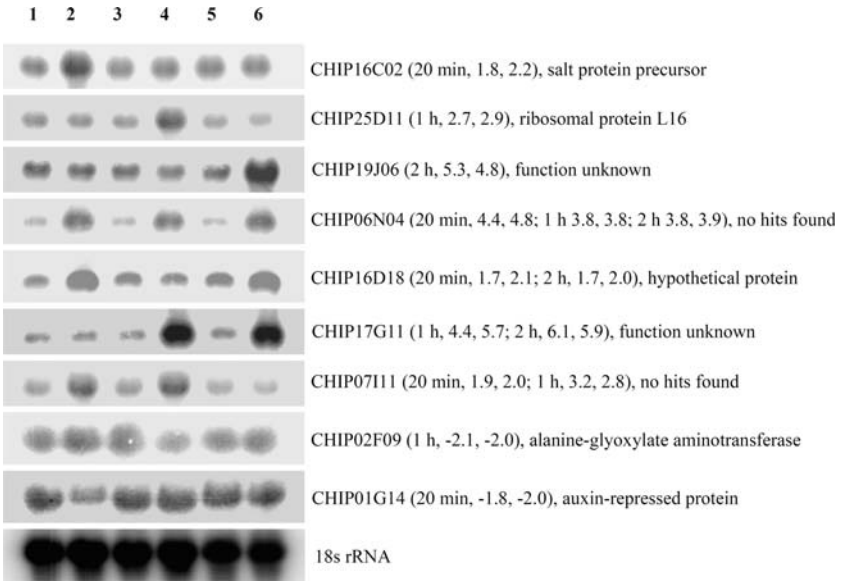

Figure 2. Comparison of the microarray data and RNA gel-blot analysis for 9 genes. Total RNA (15 μg lane^{-1}) from roots at 20 min, 1 and 2 h after low N stress and control plants were loaded and hybridized with each of the probes. The 18s rRNA blot at the bottom serves as the total RNA loading control. Lane 1, 20 min control; lane 2, 20 min stress; lane 3, 1 h control; lane 4, 1 h stress; lane 5, 2 h control; lane 6, 2 h stress. The numbers in parentheses following the time of treatment are the signal ratios of stress/control from the microarray and northern bolt respectively.

genes, 2 and 356 genes showed decreased expression at 20 min and 1 h respectively, while no difference was detected 2 h after stress. The expression data of the up- and down-regulated genes are available in the supplementary material (Table S1).

Seven genes showed elevated expressions both at 20 min and 1 h, 10 genes at both 1 and 2 h, and 5 genes at 20 min and 2 h (Table 2, Figure 4). Moreover, three of the genes were up-regulated at all three time points. None of the down-regulated genes was simultaneously detected at two different time points, while two genes were oppositely regulated at two different time points, one is CHIP01M20 encoding a hypothetical protein and the other is CHIP12L07 encoding a putative protein. This phenomenon was also observed in a phosphate starvation study in *Arabidopsis* (Hammond *et al*., 2003; Wu *et al*., 2003).

Functional classification of the differentially expressed genes

The genes that showed differential expression after low N stress grossly fell into four classes by BLASTX analysis based on sequence homology of the gene products with some of the short sequences referenced to the full length cDNAs of Kikuchi *et al*. (2003): those that had homology with genes of known functions; those that currently had unknown functions including ones annotated as expressed protein, hypothetical protein, putative protein or unknown protein; those that had homology with sequences in public databases with the E value larger than E−05 which caused credibility problem in annotations and hence were also classified as function unknown; and those that did not find hits in the protein databases. A total of 251 genes including 56 up-regulated and 195

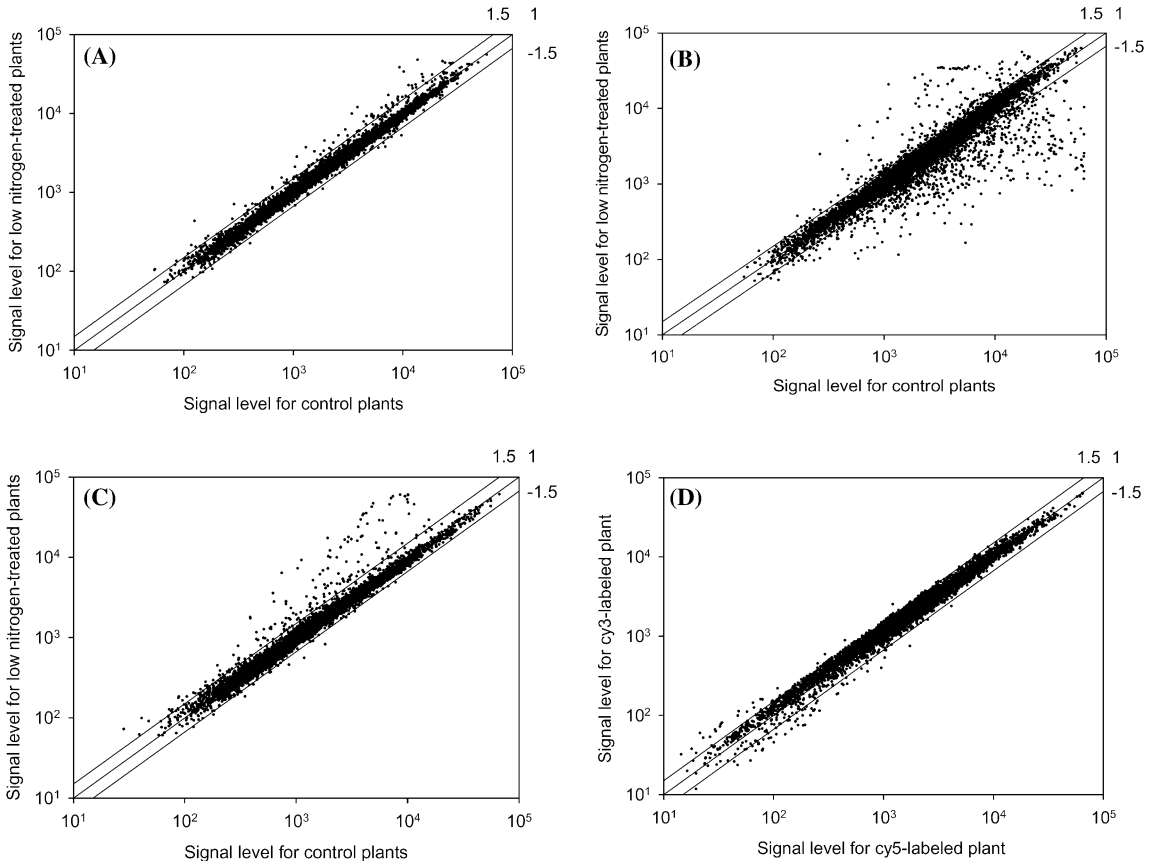

Figure 3. Scatter plots of signal intensities of low N stressed against control plants at 20 min (A), 1 h (B) and 2 h (C) of the treatment, along with the "yellow experiment" (D). Values are averaged from all the replicates. Guidelines indicate signal ratios of 1 (no change), 1.5 (induced), and −1.5 (repressed).

Table 2. Genes that were identified as showing up-regulation at two or more time points.

ID on the chips	Signal ratio (stress/control)	Gene description	Accession	Score value	E value	Functional category
20 min and 1 h						
CHIP01N06	1.6; 1.6	Function unknown	AAM00974	36.6	8.6E−02	Function unknown
CHIP07I11	1.9; 3.2	No hits found				No hits found
CHIP17F17	1.8; 1.9	Function unknown	AAB53810	41.6	7.0E−03	Function unknown
CHIP26L04	1.6; 1.8	Type−1 pathogenesis-related protein – barley	AAM93438	125	6.0E−29	Cell rescue and defense
20 min and 2 h						
CHIP16D18	1.7; 1.7	Hypothetical protein [Oryza sativa]	AAK43502	221	2.0E−57	Expressed or predicted protein
CHIP20P22	1.8; 7.7	No hits found				No hits found
1 and 2 h						
CHIP16A13	5.9; 4.0	Function unknown	EAI55684	33.1	5.0E−04	Function unknown
CHIP16D16	7.2; 4.0	Hypothetical protein	S59084	70.5	1.0E−11	Expressed or predicted protein
CHIP17A24	8.4; 10.2	Function unknown				Function unknown
CHIP17F04	4.4; 5.3	Function unknown	EAC20221	37	7.2E−01	Function unknown
CHIP17G11	4.4; 6.1	Function unknown	AAK72281	34.7	7.9E−01	Function unknown
CHIP22E13	6.0; 6.1	Hypothetical protein	BAB44102	174	1.0E−43	Expressed or predicted protein
CHIP26C14	8.2; 6.9	Putative Proline synthetase	AAM61322	294	4.0E−79	Metabolism
20 min, 1 and 2 h						
CHIP02B07	1.7; 2.0; 1.9	Salt gene product	AAB53810	294	6.0E−79	Cell rescue and defense
CHIP02B10	1.7; 1.9; 1.8	Small subunit ribosomal protein S28	CAA10101	99	3.0E−20	Protein synthesis
CHIP06N04	4.4; 3.8; 3.8	No hits found				No hits found

The gene description, accession number and the similarity statistics of each gene are based on the results of GenBank search with some of the clones referenced to the full length cDNAs of Kikuchi *et al.* (2003).

down-regulated genes belong to the first class, 77 down-regulated genes and 19 up-regulated genes belong to the second class, 59 down-regulated genes and 23 up-regulated genes belong to the third class, and 27 down-regulated and 17 up-regulated genes fall into the fourth class.

Three major classes of genes made up more than one-half of the 195 down-regulated genes with known functions: metabolism (49, 25.1%), photosynthesis (34, 17.4%) and transport facilitation (20, 10.3%) (Table 3). Of the 49 metabolism related genes, 34 were involved in carbohydrate metabolism, 8 in amino acid metabolism, 3 in fatty acid metabolism, and 4 in secondary metabolism. Among the 20 gene products functioning in transport facilitation, 10 were electron transporters, 4 ion transporters, 2 protein transporters and 4 others. Other significant functional classes of

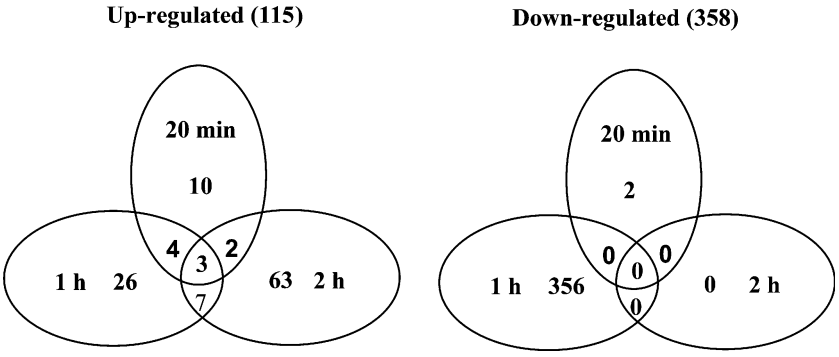

Figure 4. Number of low nitrogen stress responsive genes in rice roots detected at the three time points.

down-regulated genes included cell rescue and defense (19, 9.7%), protein synthesis (15, 7.7%), signal transduction (11, 5.6%), and transcription factors (6, 3.1%). In addition, 34 down-regulated genes with homology to known functions were unclassified.

Among the 56 up-regulated genes with homology to genes of known functions, 15 (26.8%) were involved in cell rescue and defense, 9 (16.1%) in metabolism, 6 (10.7%) in transport facilitation, and 5 (8.9%) in signal transduction. The 15 cell rescue and defense gene products included 5 salt-induced proteins, 2 metallothionein-like proteins, 2 senescence-associated proteins, 2 pathogenesis-related (PR) proteins and 4 other proteins. The 6 gene products classified as transport facilitation included electron transporters, ion transporters, protein transporters and lipid transporters. The 5 genes for signal transduction included 3 protein kinases, 1 protein phosphatases and 1 calmodulin-binding protein. Other significant functions of the up-regulated genes included transcription factors (5), protein synthesis (5), and protein destination (1). Moreover, 10 up-regulated genes with known functions were not classified into any of the functional classes.

Down-regulation of genes involved in photosynthesis by low N stress

Of the 34 down-regulated gene products for photosynthesis, 2 were chloroplast inner envelope proteins, 10 were chlorophyll *a*/*b* binding proteins, 2 were subunits of the photosystem I reaction center, 4 were photosystem II low MW protein or polypeptide or assembly factors, and 3 were involved in chlorophyll biosynthesis. Photosystems I and II, both of which are of large membrane-spanning protein–pigment complex, energize and transfer the electrons and oxidize water molecules. Reaction center, a pigment–protein complex, is a membrane that mediates the conversion of light energy to chemical energy. The expressions of the 10 genes for chlorophyll *a*/*b* binding proteins were down-regulated by 1.7–22.0-folds, and the expressions of 4 photosystem II genes were down-regulated by 2.5–20.7-folds.

The analysis also detected down-regulation of 11 clones representing 8 genes for ribulose-disphosphate-carboxylase (Rubisco) that catalyses the carboxylation of ribulose-1,5-biosphosphate to form two molecules of glycerate-3-phoshpate, the first step of dark reaction in photosynthesis. Rubisco, a complex molecule composed of eight large subunits and eight small subunits, is a pacemaker enzyme of the Calvin cycle in photosynthesis. It constitutes about 30% of the total protein in many leaves and is often described as the world's most abundant enzyme and for which reason it is of considerable interest in N nutrition of plants. In our study, the expressions for 5 of the genes for Rubisco were down-regulated by more than 20-folds and 1 of them was repressed by 53.8-folds. We also identified 2 genes for activases of Rubisco whose gene expressions were down-regulated; both of them were repressed by more than 30-folds. Other identified repressed important enzymes in the Calvin cycle included phosphoglycerate kinase, glyceraldehydes-3-phosphate dehydrogenase (NADPH chloroplast precursor),

Table 3. Functional categories of the genes with known functions as identified by microarray analysis.

Functional category	Up-regulated genes		Down-regulated genes	
	Number	%	Number	%
Cell rescue and defense	15	26.8%	19	9.7
Metabolism	9	16.1	49	25.1
Photosynthesis	0	0.0	34	17.4
Protein destination	1	1.8	5	2.6
Protein synthesis	5	8.9	15	7.7
Signal transduction	5	8.9	11	5.6
Transport facilitation	6	10.7	20	10.3
Transcription factor	5	8.9	6	3.1
Development	0	0.0	2	1.0
Unclassified	10	17.9	34	17.4

fructose-bisphosphate aldolase (chloroplast precursor) and glucose-6-phosphate isomerase.

In the Calvin cycle, the carboxylation of 3 molecules of ribulose-1, 5-biosphosphate (RuBP) leads to the net synthesis of one glyceraldehydes-3-phosphate molecule and regeneration of 3 molecules of RuBP. Some important enzymes in the reactions of RuBP regeneration were also down-regulated, including sedoheptulose-1, 7-bisphosphatase, ribulose-5-phosphate-3-epimerase and ribulose-5-phosphate kinase.

Additionally, the analysis also identified 2 genes for enzymes in starch synthesis, the starch branching enzyme RBE4 and granule-bound starch synthase, whose expressions were repressed 2.0 and 9.1-folds respectively.

Down-regulation of energy metabolism genes by low N stress

Nitrate assimilation, ammonium assimilation and amino acid biosynthesis are very energy consuming (Werf *et al*., 1988). Carbohydrate metabolism provides both the energy required in the above processes in the form of the reducing equivalents (NADH and Fd), and the organic acid 2-oxoglutarate as the primary ammonium acceptor in the GS/GOGAT pathway (Kaiser *et al*., 2000).

In glycolysis, a biochemical pathway that occurs almost in every living cell, each glucose molecule is split and converted to 2 molecules of pyruvate. The energy captured during glycolytic reactions is stored in two molecules, ATP and NADH. It was detected that expressions of genes for glyceraldehyde−3-phosphate dehydrogenase, fructose-bisphosphate aldolase and fructose−6-phosphate−2-kinase, 3 key enzymes in the glycolysis pathway, were repressed.

Fd (Ferredoxin) is a reductant required in nitrite reduction and in ammonium assimilation by Fd-GOGAT. In photosynthetic leaves, Fd is reduced in photosystem I, and in root, Fd is reduced in plastids by FNR (Fd $NADP^+$ oxidoreductase) using NADPH as the reductant. NADPH is produced by the hexosephosphate shunt pathway, which converts glucose 6-phosphate into ribose 5-phosphate and reduces two NADPH molecules. It was reported that the activity of 6-phosphogluconate dehydrogenase (6-PGDH), a key enzyme in this pathway, is strongly induced by nitrate in maize (Redinbaugh and Campbell, 1998), and the gene expression is nitrate inducible in *Arabidopsis* (Wang *et al*., 2000). In our study, this gene was repressed by 2.0-folds with low N stress.

Both up- and down-regulations of biotic and abiotic stress responsive genes by low N stress

Higher plants are constantly challenged with biotic and abiotic stresses imposed by the environments. In responses to these stress conditions, plants have evolved a diversity of adaptive physiological and biochemical mechanisms. These include cell wall lignification, synthesis of antimicrobial low-mass compounds (phytoalexins), and the expression of a number of genes.

PR proteins are evolutionarily conserved in the plant kingdom and induced by biotic or abiotic pathogenic agents (Dixon and Lamb, 1990; Linthorst, 1991). Many PR proteins also display enzymic activities, such as chitinase, β−1, 3-glucanase, protease activities (Kauffmann *et al*., 1987; Legrand *et al*., 1987), as well as other putative antimicrobial activities (Geoffroy *et al*., 1990).

We identified two genes for PR proteins: type-1 pathogenesis-related protein and pathogenesis-related protein 10, with the expressions up-regulated by 1.6 and 1.8-folds respectively. Another three PR proteins, a putative thaumatin-like pathogenesis-related protein, a pathogenesis-related protein class 1, and a pathogenesis-related protein 4b were down-regulated by 3.8, 2.2 and 1.7-folds, respectively. In addition, 2 genes encoding class IV chitinase (CHIV) and class III chitinase were also down-regulated by 3.7 and 10.2-folds, respectively.

It is well established that many environmental stressors such as salinity, drought, temperature or heavy metals cause a rapid and excessive accumulation of reactive oxygen species (ROS) in plant cells, which form the oxidative stress to the plant and are regarded as a major cause of cellular damage and cell death (Allen, 1995; Mittler, 2002). The results of a recent report indicated that ROS were also involved in responses to nutrient deficiency (Shin and Schachtman, 2004).

The expression profiling identified one gene for aldehyde dehydrogenase whose expression was induced by 2.1-folds after low N stress for 2 h. Aldehydes are major products of lipid peroxidation caused by ROS and are toxic to the cell,

because of their chemical reactivity. Many aldehydes are oxidized by the large family of $NAD(P)^+$ dependent aldehyde dehydrogenases. This large enzyme family consists of diverse subfamilies with four different functions: detoxification, osmotic protection, intermediary metabolism and NADPH generation (Perozich *et al*., 1999). Indeed, Sunkar *et al*. (2003) overexpressed aldehyde dehydrogenase *Ath-ADH3* in *Arabidopsis*, which improved the tolerance of transgenic lines when exposed to dehydration, NaCl, heavy metal, methyl viologen and H_2O_2.

The analysis also identified two genes for the catalase isozyme, one is down-regulated by 2.0-folds and the other is up-regulated by 2.1-folds. Catalase is a heme-containing enzyme that converts H_2O_2 to oxygen and water, which also plays an important role in detoxification of ROS that are generated by various environmental stresses (Scandalios, 1990). Many higher plants have multiple isoforms of catalase. In maize three distinct genes encode the catalase isoenzymes whose expressions are regulated differentially in response to changes in environmental conditions or developmental phase (Redinbaugh *et al*., 1988; Scandalios, 1990). Two isoenzymes of cotton catalase also exhibited different expression patterns (Ni and Trelease, 1991). In rice 2 genes for catalase have been cloned and they showed distinct expression patterns (Mori *et al*., 1992; Morita *et al*., 1994).

A gene encoding proline synthetase was identified in our microarray analysis whose expression was up-regulated by 8.2-folds after low N stress for 1 h and remained high at 2 h after the treatment. Proline has a role in stress tolerance as a compatible osmolyte and osmoprotectant, which has been extensively studied (Yancey *et al*., 1982). Okuma *et al*. (2000) found that exogenous proline improved the growth of salt-stressed tobacco cell culture, by acting as an osmoprotectant for enzymes and membranes against salt inhibition. Nanjo *et al*. (1999) found that, in addition to as compatible osmolyte and osmoprotectant, proline also induced the synthesis of key proteins that are necessary for stress responses.

Metallothionein-like protein, senescence-associated protein, salt-induced protein are frequently found in stress responses (Steffens, 1990; Buchanan-Wollaston, 1994; Hirano *et al*., 2000). We identified 2 genes encoding metallothionein-like proteins, 5 genes encoding salt-induced proteins and 2 genes encoding senescence-associated proteins with the expressions elevated by 1.6–3.4-folds. However, it was also found that 2 other genes for metallothionein-like proteins and 1 gene for senescence-associated protein were down-regulated, with the level of expression reduced by 1.9–10.3-folds.

Both up- and down-regulation of regulatory genes by low N stress

In response to alterations in the environment, signal transduction pathways target transcription factors, transcriptional regulators and chromatin modifying factors leading to their phosphorylation by protein kinases or dephosphorylation by protein phosphatases. These modifications either positively or negatively influence transcription factor activity to regulate gene expression network resulting in appropriate changes in cell behavior (Hunter and Karin, 1992; Hill and Treisman, 1995). Protein decoration of phosphorylation and dephosphorylation can directly regulate distinct aspects of transcription factor function, including cellular localization, protein stability, protein–protein interactions and DNA binding. The phosphorylation-dependent modulation of the activities of transcriptional coregulators and chromatin-modifying factors can also control transcription factor activity (Whitmarsh and Davis, 2000).

In this study, 5 genes involved in signal transduction were identified as up-regulated by low N stress, including 3 protein kinase genes induced by 1.7–2.3-folds, 1 protein phosphatase genes induced 5.5-folds, and 1 gene for putative calmodulin-binding protein induced by 1.6-folds. Another 11 genes involved in signal transduction were down-regulated by low N stress, including 6 protein kinase genes with the expression level reduced by 2.0–33.6-folds, 4 protein phosphatase genes reduced by 1.6–3.7-folds and 1 gene for unknown protein reduced by 12.7-folds.

A number of families of transcription factors, such as AP2/EREBP, bZIP/HD-ZIP, Myb, and several classes containing zinc finger domains, have been implicated in plant stress responses because their expression is induced or repressed under different stress conditions (Shinozaki and Yamaguchi-Shinozaki, 2000; Chen *et al*., 2002).

Altering the expression of certain transcription factors can greatly influence plant stress tolerance (Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999).

We identified 5 transcription factor genes whose expressions were induced by 1.9–9.3-folds: 1 myb family transcription factors, 1 probable sigma-like transcription initiation factor, 1 RING-H2 finger protein, 1 putative G-box binding factor 8 and 1 putative transcription factor. Six genes for transcription factors were down regulated by 1.6–2.9-folds: 1 heat shock factor, 1 bHLH family protein, 1 zinc finger family protein, 1 sigma factor, 1 putative MYC transcription factor and 1 auxin response factor.

Little response of genes involved in N assimilation pathways to low N stress

The pathways of nitrogen uptake and assimilation and the gene products involved in the pathways have been well documented (Hirel and Lea, 2001). In our microarray slides, there are 1 cDNA clone for nitrate transporter (CHIP23D08), 1 clone for NR (CHIP24E18), 4 clones for GS (CHIP01H09, CHIP20I11, CHIP21H15, and CHIP04M08), 2 clones for GOGAT (CHIP17L03, CHIP10N20), 2 clones for GDH (CHIP04G05, CHIP18O19), and 3 clones for AspAT (CHIP01A21, CHIP07N15, CHIP09N06). The expressions of all these clones, except one (CHIP04M08) for chloroplastic GS2 whose expression was repressed by 1.7-folds, were essentially unaffected by the low N stress.

Discussion

The microarray analysis of early response of rice seedlings to low N stress at three time points after the treatment with a total of 11,494 cDNAs likely representing 10,422 unique genes from a normalized cDNA library detected differential expression of 471 genes, with 158 of them showing elevated expression and 355 showing reduced expression. Although the functions for about 47% of the differentially expressed genes cannot be deduced at present based on sequence homology to genes of known functions, the analysis clearly revealed the following characteristics of the early response of the rice seedling to low N stress: (1) the genes involved in photosynthesis and energy metabolism were down-regulated rapidly, (2) many of the genes involved in early responses to biotic and abiotic stresses were up-regulated while many other stress responsive genes were down-regulated, (3) regulatory genes including transcription factors and ones involved in signal transduction were both up- and down-regulated, and (4) the genes known to be involved in N uptake and assimilation showed little response to the low N stress.

It is understandable that, when encountering N deficiency, the plant has to shut down the energy and nutrient consuming activities such as photosynthesis and TCA cycle in order to survive, as an adaptive mechanism. An interesting feature yet to be understood is that the large number of genes for the photosynthetic apparatus such as proteins and enzymes that function specifically in the chlorophyll were expressed in root, a phenomenon that was also observed in gene expression profiling in *Arabidopsis* (Himanen *et al*., 2004), while the expressions of these genes were little affected in the leaves. This suggested that the mild stress of the low N treatment at the early stage sensed by roots has not affected the leave tissues.

The second characteristic seems to indicate extensive cross-talk between the pathways of responses to low N stress and reactions to pathogen attack and abiotic stresses, suggesting that there may be substantial physiological similarities between low N stress and other biotic and abiotic stresses, as sustained by the plant. The up-regulations of the genes indicate that those genes may play important protective roles against low N stress, while down-regulations of the important genes for responses to biotic and abiotic stresses, although possibly functioning as a strategy to survive, may make the plants more vulnerable to those stress conditions.

The rapid alterations of the expression levels of the regulatory elements, such as genes involved in signal transduction and transcription regulation, represent the primary response of the regulatory machinery to the low N stress. The patterns of both induced and repressed expression exhibited by the regulatory elements are consistent with the up- and down-regulations of functional genes of various classes identified, suggesting a cohesive nature of the expression network. Although it is not possible to configure such a complex regulatory network based on the expression data obtained in this study, understanding the changes in the regulatory elements may be the key points for understanding the response to low N stress.

It seems surprising that most of the genes arrayed in the chips known to play key roles in N uptake and assimilations did not show significant response to the low N stress. This is probably due to the fact that the samples were taken at the early stage of the low N stress and the N level of the treatment of 0.24 mM NH_4NO_3 in the solution was not severe enough to cause immediate N shortage as the substrate for these enzymes, although it could lead to the development of chlorosis of leaves at later stages of the low N stress treatment (data not shown).

The challenges for future studies are to characterize functional roles in relation to N metabolism of the genes that are responsive to low N stress, including the large number of genes presently with unknown functions. Over expression and RNA interference have demonstrated to be useful strategies for determining the functions of the genes, which will be used in our future work for characterizing the genes identified in this study. In this connection, it is interesting to note that a number of the low N stress inducible genes are located in the genomic regions where QTLs for NUE were detected by genetic mapping (Lian XM *et al*., unpublished data), which may also provide clues to the understanding of genes and mechanisms underlying the low N stress response and also for the improvement of NUE of the rice crop.

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